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Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes

Reinvestigation of assay conditions

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Abstract. Assay conditions in determining total cytochrome P-450 in four laboratories were compared. Although the determination was derived from the original Omura and Sato method in each laboratory, the four standard protocols differed slightly, resulting in considerable differences in the results. Since the cytochrome P-450 content is usually expressed per mg protein, the protein assay conditions were evaluated as well. Furthermore, we compared the cytochrome P-450 values obtained by the CO- and the dithionite (DT)-difference methods. The effect of a number of variables in the assay was investigated. The influence of the storage temperature of the microsomes was ascertained as well as effects of the gassing time with CO and the time between addition of dithionite, CO-gassing and the recording of the difference spectra. After evaluating these variables a standard operation procedure was established. Using this procedure the interlaboratory coefficient of variation for total cytochrome P-450 was 4.8%, a value which was comparable to the intralaboratory coefficients of variation. The final results also show that the millimolar extinction coefficient for the DT-difference method is higher than for the CO-difference method.

Key words: Cytochrome P-450 determination – Protein determination – Liver microsomes – Interlaboratory comparison

Introduction

The endoplasmatic reticulum of the liver and many other tissues contain a number of different cytochrome P-450s which perform a central role in the metabolism of xenobiotics and various endogenous compounds. Measurement of total cytochrome P-450 content in the liver or cultured hepatocytes is therefore frequently included in toxicological and pharmacological studies. The methods for the determination of total cytochrome P-450 are usually based on measurement of the Soret peak at 450 nm of the carbon monoxide (CO)-adduct of cytochrome P-450. The protocols for the measurement are generally derived from the method described by Omura and Sato (1964a, b). Cytochrome P-450 contents can be determined using either the CO-difference spectrum or the dithionite (DT)-difference spectrum. In the CO-difference method the microsomal suspension in sample and reference cuvette is first reduced with DT, after which the microsomes in the sample cuvette are gassed with CO. In the dithionite method microsomes in the sample and reference cuvette are treated with CO first, then the sample is reduced with DT. The latter method was used inter alia by Schoene et al. (1972).

Each of the four laboratories participating in this study developed its own protocol for the determination of total cytochrome P-450 in liver or cultured hepatocytes, based on the method of Omura and Sato (1964a, b). Since these protocols differed in a number of assay conditions, we were interested in the extent to which the different protocols affected the results of the cytochrome P-450 assay. Furthermore, it was felt desirable to develop a common protocol to make the results of the four laboratories comparable. Therefore, a program was set up to compare the individual protocols and to develop a common, more optimized method. The program also included comparison of the cytochrome P-450 values calculated from the CO- and the DT-difference spectra, based on the different millimolar extinction coefficients for both P-450 complexes, i.e. 91 $mM^{-1}.cm^{-1}$ for the CO- and 104 $mM^{-1}.cm^{-1}$ for the DT-difference spectra as published by Omura and Sato (1964b) and Matsubara et al. (1976).

The cytochrome P-450 content is frequently expressed as nmol cytochrome P-450 per mg protein. Therefore, the assay protocols for the protein determination by the Lowry method (1951) of each laboratory were compared as well and a common protocol developed.

The objectives of this study were: (a) to compare the original methods for determination of total cytochrome P-450 and protein in the participating laboratories, (b) to reinvestigate a number of assay conditions, and (c) to develop a common, optimized protocol. This would enable a more direct comparison of total cytochrome P-450 and protein values obtained in toxicological experiments carried out in the participating laboratories.

Materials and methods

Chemicals. Phenobarbital (sodium salt) was purchased from BDH Chemicals Ltd, Poole, England, bovine serum albumin (BSA) from Sigma Chemical Comp., St Louis, USA, and Preciset from Boehringer, Mannheim, FRG. All other chemicals used were of analytical grade.

Animals and housing. Three-month old male Wistar rats (Wistar, CPB:WU random) were obtained from the TNO

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Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands. The rats were allowed free access to food and water. The animals were caged in wire mesh cages at 22 ± 1° C on a 12 h light/dark cycle and a relative humidity of >40%.

Phenobarbital treatment. Phenobarbital (0.1%) was administered in the drinking water for 7 days. Fresh phenobarbital solutions were prepared daily.

Preparation of microsomes. Livers were perfused in situ via the portal vein with a 0.9% NaCl solution till the entire liver was yellow, while the animals were under ether anaesthesia. The livers were excised, weighed, collected in ice-cold 1.15% KCl, sliced and homogenized in 3 volumes ice-cold 1.15% KCl using a Potter-Elvehjem glass teflon homogenizer. The cell debris, nuclei and mitochondria were removed by centrifugation for 20 min at 9000 *g* in a Runne 108E-K-Ti refrigerated (0–4° C) centrifuge. The floating fat layer was carefully removed and the underlying supernatant fraction decanted and homogenized. The post-mitochondrial fraction was centrifuged at 105000 *g* for 75 min at 0–4° C in a Beckman L8-70 ultracentrifuge. The pellet was resuspended carefully with an ultra turrax in ice-cold 1.15% KCl. The final weight of the microsome suspension was equal to the original liver weight.

Experimental procedure. Four series of rats were used. In the first experiment the cytochrome P-450 content was measured in liver microsomes from ten control and ten phenobarbital-treated rats individually. In the other three experiments, mixtures of pooled microsomes from four treated and four untreated rats were used in order to obtain five levels of cytochrome P-450. Five mixtures were prepared containing 100% control (Pool 1), 75% control and 25% phenobarbital (Pool 2), 50% control and 50% phenobarbital (Pool 3), 25% control and 75% phenobarbital (Pool 4), and 100% phenobarbital (Pool 5) microsomes. Samples of 1 ml were quickly frozen in liquid nitrogen and stored in closed vials at −80° C or −20° C prior to use. The samples were transported to the participating laboratories in containers with solid CO_2.

Spectrophotometers. Laboratory A used a Beckman DU8, laboratory B a Pye-Unicam SP 1800, laboratory C a Pye-Unicam SP8-400, and laboratory D a Perkin Elmer Lambda 5 spectrophotometer.

Cytochrome P-450 determination. Cytochrome P-450 was determined according to the method of Omura and Sato (1964a, b) for the CO-difference spectrum and to Schoene et al. (1972) for the DT-difference spectrum. All determinations were carried out in triplicate. In all laboratories determinations were performed within the same week. The expression of the total cytochrome P-450 content was based on the difference in absorbance between 450 nm and 490 nm wavelength and converted to a concentration using a millimolar extinction coefficient of 91 $mM^{-1}.cm^{-1}$. The recorded spectra showed no absorbance maxima at a wavelength of about 420 nm, which indicates that the microsomal preparations did not contain detectable amounts of carboxyhaemoglobin or denatured cytochrome P-450. In the DT-difference spectra a small peak was observed at 425 nm, which disappeared after addition of NADH, indicating the presence of cytochrome b5. The different methods applied in the laboratories at the beginning of this study are listed in Table 1. The final assay conditions used in the interlaboratory comparison for the determination of the cytochrome P-450 content in rat liver microsomes are listed in Table 3.

Table 1. Assay conditions of the cytochrome P-450 determination in rat liver microsomes in laboratories A, B, C and D: *starting conditions*

Conditions and reagents	Laboratories			
	A	B	C	D
Microsome dilution	10 times	20 times	20 times	20 times
Buffer molarity	0.1 M	0.08 M	0.0525 M	0.1 M
Buffer (pH = 7.4)	phosphate (Sörensen)	phosphate (Sörensen)	tris. HCl in 77 mM KCl	phosphate (Sörensen)
Emulgen/sucrose	*	0.2%/1.25%	*	*
EDTA	1 mM	1 mM	*	0.1 mM
Dithiothreitol	1 mM	1 mM	*	*
Glycerol	20%	20%	30%	*
$MgCl_2$	*	*	*	5 mM
CO-gas-time	20 s	10–15 s	30 s	20 s
CO-gas-flow	1 bubble/s	1 bubble/s	1 bubble/s	1 ml/10 s
Temperature	17°C	24°C	20°C	25°C
Slit width ($\Delta\lambda$)	2 nm	2 nm	2 nm	1 nm
Dithionite addition	few crystals	few crystals	4.58 mM[a]	few crystals
CO-difference[b], t_{CO}	4 min	2 min	5 min	1 min
$t^{start}_{recording}$	4.5 min	3 min	10 min	2 min
DT-difference[c], t_{DT}	0.5 min	1–2 min	1–3 min	1 min
$t^{start}_{recording}$	4 min	3–4 min	6–8 min	2 min

* Not added to the assay medium, t_{CO} = time CO-gassing, t_{DT} = time dithionite addition and $t^{start}_{recording}$ = starting time of recording the difference spectrum

[a] 10 µl 1.14 M (stored on ice) added to 2.49 ml assay medium, a fresh solution was made every hour

[b] Carbon monoxide (CO) difference spectrum with t_{DT} = 0 min

[c] Dithionite (DT) difference spectrum with t_{CO} = 0 min

Table 2. Assay conditions of the protein determination in rat liver microsomes in laboratories A, B, C and D: *starting conditions*

Conditions and reagents	Laboratories			
	A	B	C	D
Microsome dilution	600 times	650 times	670 times	975 times
Na_2CO_3	0.142 M	0.138 M	0.207 M	0.142 M
NaOH	75 mM	73 mM	73 mM	75 mM
Na-K-tartrate	0.53 mM	*	1.04 mM	0.53 mM
Na-citrate	*	0.26 mM	*	*
$CuSO_4 \cdot 5\,H_2O$	0.30 mM	0.15 mM	0.59 mM	0.30 mM
Na-desoxycholate	*	*	0.33%	*
SDS[a]	*	0.05%	*	*
Folin 1 M	250 µl	200 µl	500 µl	250 µl
Final volume	6.50 ml	2.60 ml	6.70 ml	3.25 ml
Wavelength	660 nm	758 nm	750 nm	725 nm
Reaction time 1[b]	10 min	10 min	5–10 min	10 min
Reaction time 2[c]	30 min	30 min	30 min	30 min
Protein standard	BSA[d]	BSA	BSA	Preciset

* Not added to the assay medium
[a] Sodium dodecyl sulphate
[b] Incubation time after addition of Lowry C (see Materials and methods)
[c] Incubation time after addition of Folin
[d] Bovine serum albumin

Table 3. Assay conditions of the cytochrome P-450 in rat liver microsomes in laboratories A, B, C and D: *final conditions*

Parameters	Values
Microsome dilution[a]	20 times
Buffer molarity[b]	0.1 M
Buffer	phosphate (Sörensen)
pH	7.4
Glycerol	20%
CO-gas time	30 s
CO-gas flow	5 bubbles/s
Temperature	20°C
Slit width ($\Delta\lambda$)	1 nm
Dithionite addition	4.58 mM[c]
CO-difference[d], t_{CO} =	2 min
$t^{start}_{recording}$ =	at least 7 min
DT-difference[e], t_{DT} =	2 min
$t^{start}_{recording}$ =	at least 7 min

[a] Concentrated microsomes stored at −80°C were placed in a waterbath (37°C) for 4 min and kept on ice until use
[b] 800 ml 0.125 phosphate buffer + 200 ml glycerol, t_{CO} = time CO-gassing, t_{DT} = time dithionite addition and $t^{start}_{recording}$ = starting time of recording the difference spectrum
[c] 10 µl 1.14 M (stored on ice) added to 2.49 ml assay medium, a fresh solution was made every hour
[d] Carbon monoxide (CO) difference spectrum with t_{DT} = 0 min
[e] Dithionite (DT) difference spectrum with t_{CO} = 0 min

Protein determination. The protein content in rat liver microsomes was determined according to the method described by Lowry et al. (1951). The starting conditions are listed in Table 2 and the final conditions applied in this study are described below.

The Lowry A solution contained: 20.0 g $Na_2CO_3.l^{-1}$ (0.19 M), 4.0 g $NaOH.l^{-1}$ (0.1 M) and 0.2 g Na-K-tartrate.l^{-1} (0.7 mM), and the Lowry B solution: 5.0 g $CuSO_4.5\,H_2O.l^{-1}$ (20 mM). Lowry C, a mixture of 50 parts Lowry A and 1 part Lowry B, was freshly prepared. The Folin reagent was diluted to 1 M. The microsomal suspensions were diluted 150 times in distilled water. Serial dilutions were made of BSA containing: 0.04, 0.08, 0.12, 0.16, 0.20 and 0.24 $g.l^{-1}$. Of the diluted microsome suspension (or BSA solution) 500 µl were added to a plastic tube together with 2.5 ml Lowry C. The solution was thoroughly mixed. After 10 min 250 µl 1 M Folin reagent was added to the plastic tube and mixed immediately. The absorbance was measured 30 min after the addition of Folin at a wavelength of 750 nm. The protein contents of the microsomal suspensions were calculated using the BSA curve.

Statistics. The curves shown in Figs. 2, 3, 5, and 6 were obtained by calculating the best fitting lines using the least squares method. Mean coefficients of variation (CV) within each laboratory were calculated in the first experiment using mean cytochrome P-450 values (as ΔE) or protein values ($g.l^{-1}$) obtained from microsomes of ten control rats and ten phenobarbital-treated rats. In the final experiment CVs were calculated using mean cytochrome P-450 or protein values from the five microsome pools. The correlation between the results of the CO- and DT-difference methods was calculated by the method of Pearson (1981) and tested by Student's *t*-test.

Results and discussion

A comparison of the total cytochrome P-450 content in rat liver microsomes prepared from ten control and ten phenobarbital-treated animals determined in the four participating laboratories (A, B, C, D) by their own methods is presented in Fig. 1. The mean cytochrome P-450 contents were obtained from the measurement of individual liver microsome preparations from control and phenobarbital animals in each laboratory using their own protocol (Table 1). Starting conditions for the protein assay used are presented in Table 2. The mean values found for microsomal cytochrome P-450 in both control and phenobarbital-treated rats showed marked differences between the

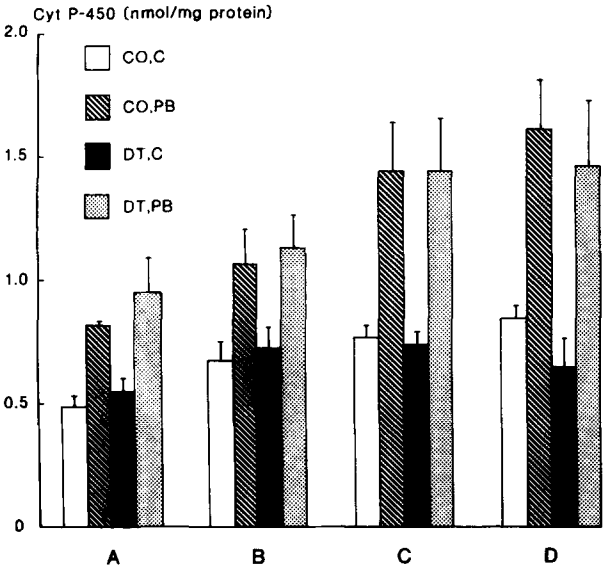

Fig. 1. Cytochrome P-450 content of rat liver microsomes prepared from untreated (*C*) or phenobarbital-treated (*PB*) animals, calculated from the carbon monoxide (*CO*) or the dithionite (*DT*) difference spectra. Determinations from laboratories A–D. Data are means ± s.d. (n = 10 rats)

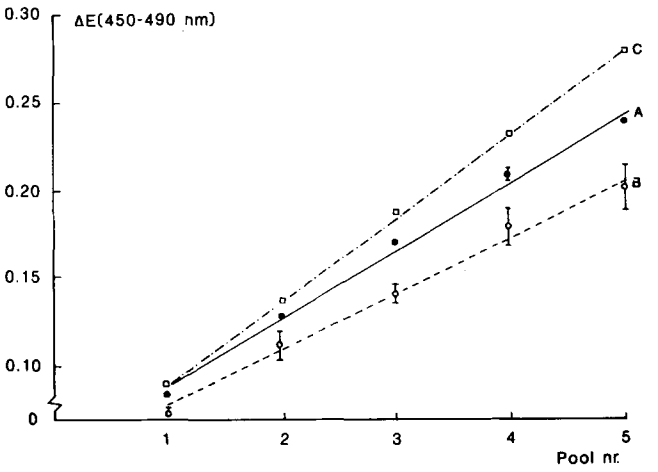

Fig. 2. Cytochrome P-450 determination (CO-difference) in rat liver microsomes from untreated and phenobarbital-treated animals and from mixed samples (Pool 1–5, see *Materials and methods*). Determinations were carried out in three different laboratories (A–C), according to a partly standardized protocol (see text). Data are means of triplicate determinations, *vertical bars* represent ± s.d.; if no bar is shown s.d. was smaller than the symbol used

four laboratories. The ratio between P-450 content of control and phenobarbital-treated rats was different as well. Minor differences were observed between the results of the CO- and DT-methods. As shown in Table 4, relatively large intralaboratory coefficients of variation (CV) were found for the cytochrome P-450 and the protein contents, and a high interlaboratory CV value (20.6%) for the cytochrome P-450 content. In spite of the high CV values (8.25–11.70%) for the protein assay within the laboratories a small CV (3.77%) was found between the laboratories.

From the results it was concluded that the differences observed were most likely due to differences in the original methods, for instance in buffer composition or gassing times (Tables 1 and 2). Therefore, a second experiment was designed to investigate a number of assay conditions in a partly standardized protocol for the cytochrome P-450 as well as for the protein assay. The assay conditions studied were the influence of storage temperature, thawing procedure, CO-gassing time, buffer composition, and the addition of dithiothreitol and EDTA. In this experiment only the CO-difference spectrum was recorded, using five microsome pools with increasing cytochrome P-450 contents (see Materials and methods). The partly standardized protocol was the same as shown in Table 3, with the following exceptions: buffer composition and microsome dilution were the same as shown in Table 1; the CO gas flow was 1 bubble.s^{-1} for 20 s and the recording of the CO-difference spectrum started 4 min after the addition of dithionite.

The results obtained for cytochrome P-450 with the new protocols are presented in Fig. 2. Small interlaboratory differences in the cytochrome P-450 content were observed in control microsomes (pool 1). Although the absolute differences increased in the microsome samples containing higher cytochrome P-450 levels, the relative differences were comparable in all pools, as expected. Addition of EDTA (0.1 or 1.0 mM) or dithiothreitol (0.5 or 1.0 mM) to the assay buffer did not affect the results of the assay (data not shown).

The effect of storage conditions on the cytochrome P-450 content in microsomes is presented in Fig. 3. No differences in cytochrome P-450 content were observed in microsomes stored at −80° C for 1 day or 4 weeks. However, storage at −20° C for 4 weeks resulted in lower cytochrome P-450 contents in comparison with storage at −80° C.

The stability and storage conditions of liver homogenates were studied by several investigators (Bartosek et al. 1980; Dent et al. 1981; Danner-Rabowsky and Groseclose

Table 4. Coefficients of variation (%) within each laboratory and between the laboratories for the cytochrome P-450 and the protein assays at the beginning and at the end of the study

Laboratory	Start of the study		End of the study	
	Cytochrome P-450[a]	Protein	Cytochrome P-450[a]	Protein
A	9.36	8.25	3.30	2.47
B	14.86	11.70	4.09	1.06
C	12.30	8.40	2.51	1.62
D	18.01	9.05	5.55	2.81
Between the laboratories	20.58	3.77	4.80	7.70

For calculation of CV see *Materials and methods*

[a] CO-difference spectrum

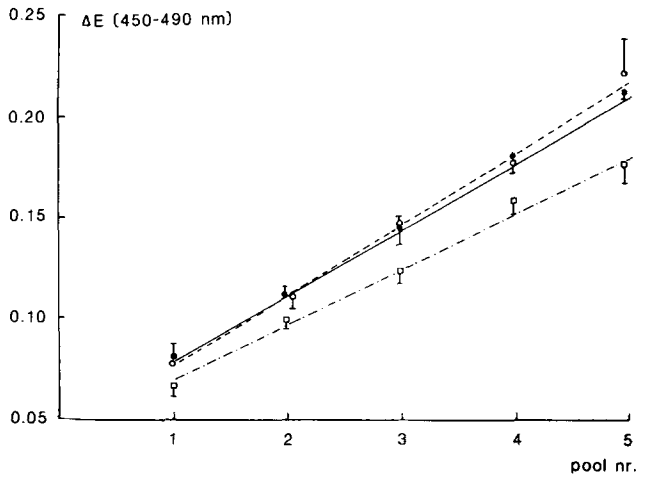

Fig. 3. The effect of storage conditions on the cytochrome P-450 (CO-difference) content in rat liver microsomes. 1–5: different pools of mixed samples of microsomes as described in *Materials and methods*. Data are means ± s.d. of triplicate determinations. (●———●): determined after 1 day storage at −80° C. (○-----○): determined after 4 wks storage at −80° C. (□-·-·-□): determined after 4 wks storage at −20° C

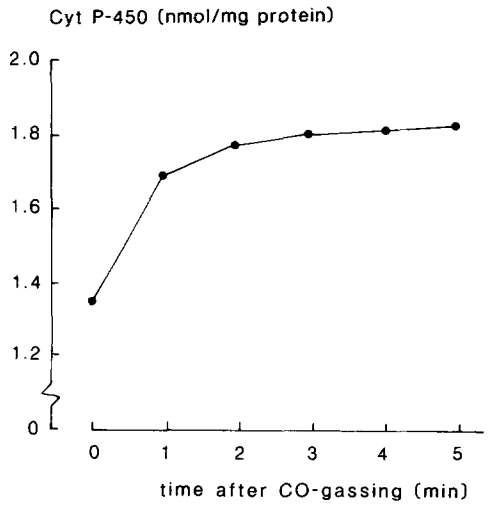

Fig. 4. The effect of time after CO-gassing on the cytochrome P-450 determination in rat liver microsomes. Four minutes after addition of dithionite, samples were gassed with CO for 20 s, 1 bubble/s. Data are means of triplicate determinations, s.d. was smaller than the symbol used

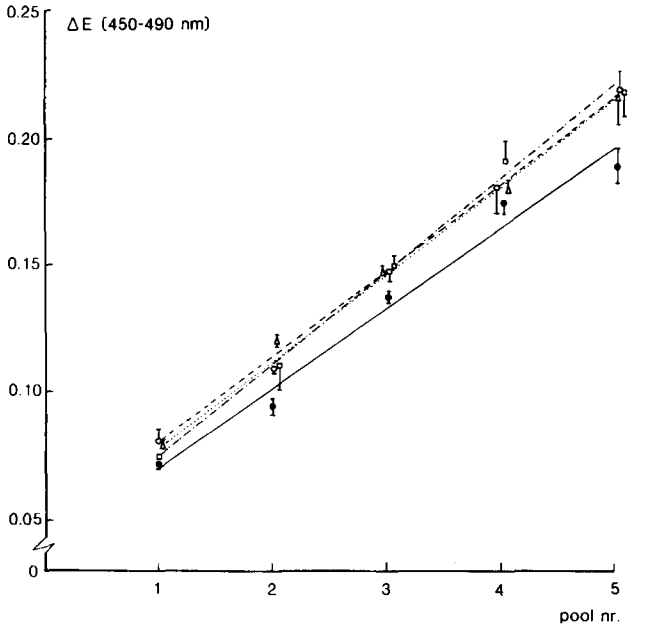

Fig. 5. Effect of buffer composition on the cytochrome P-450 determination (CO-difference) in rat liver microsomes (1–5: see *Materials and methods*). Data are means ± s.d. of triplicate determinations. (●———●): buffer + sucrose and Emulgen. (○-----○): buffer + Emulgen. (□-·-·-□): buffer + sucrose. (△·····△): buffer

1982). Bartosek et al. (1980) observed minor losses in rat liver microsomes during short-time storage at −80° C or −20° C. Results obtained with long-term storage of the microsomes showed that the half-life of total cytochrome P-450 content was about 3 years when stored at −80° C. Furthermore, Dent et al. (1981) found no effects on rat liver microsomes due to long-term storage at −85° C. The cytochrome P-450 contents declined to 70% of the initial values after 2 weeks' storage, but after 4 weeks' storage no differences from the initial cytochrome P-450 values were observed. Total cytochrome P-450 contents were not affected in liver post-mitochondrial fractions after storage at −80° C for 13 weeks (Danner-Rabovsky and Groseclose 1982). However, these authors found the apparent protein content to be doubled during storage, probably caused by loss of water from the samples during prolonged storage at low temperatures. In the present study no storage related differences with respect to the protein content of liver microsomes were found (data not shown).

Different thawing procedures (thawing slowly on ice, thawing overnight at 4° C or thawing in a water bath at 37° C for 4 min) revealed only minor effects on the results of the cytochrome P-450 determination (data not shown). Since fast thawing at 37° C resulted in somewhat higher cytochrome P-450 levels, we used this standardized thawing procedure in further experiments.

The time between CO-gassing and recording of the difference spectrum is known to be an important factor in the cytochrome P-450 determination (Hayes 1982). Therefore, the optimal time span should always be assessed in each experiment, since it may be dependent on the composition of the individual cytochromes P-450. In our samples, a time span of 3–5 min is required in order to obtain a constant, maximal level for the apparent cytochrome P-450 content (Fig. 4).

The relatively low cytochrome P-450 levels measured by laboratory B (Figs. 1 and 2) were found to be related to the buffer composition used in the assay mixture. The combination of sucrose and Emulgen in their buffer solution decreased the apparent cytochrome P-450 content as compared to a buffer containing sucrose or Emulgen separately or a buffer without these additions (Fig. 5).

The evaluation of the results obtained in the second set of experiments indicated that by applying the partly standardized protocol the differences in the results of the cytochrome P-450 determination between the various laboratories decreased. However, there were still differences in the protocols. Therefore, it was decided to perform a third and fourth interlaboratory comparison with a strictly stan-

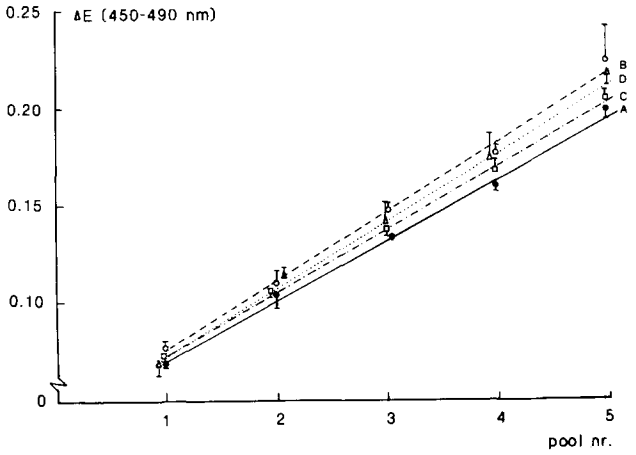

Fig. 6. Cytochrome P-450 determination as calculated from CO-difference spectra in rat liver microsomes (1–5: see *Materials and methods*). *Lines* represent determinations in laboratories A–D, according to the standard operation procedure as described in Table 3. Data are means ± s.d. of triplicate determinations

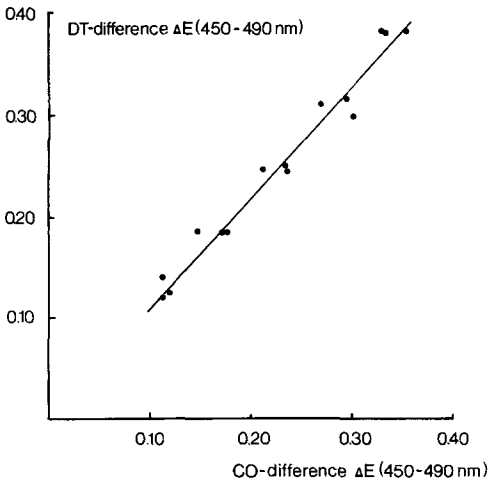

Fig. 7. Correlation between cytochrome P-450 determinations calculated from carbon monoxide (*CO*) and dithionite (*DT*) difference spectra. Determinations were carried out in rat liver microsomes (1–5: see *Materials and methods*) according to the standard operating procedure as described in Table 3. Data are means of triplicate determinations, carried out in the same laboratory on the same sample. Correlation $y = -0.0004 + 1.088x$, $p = 0.9861$, $p < 0.001$

dardized protocol, established on the basis of the second set of experiments. In particular buffer composition, addition of dithionite and incubation as well as measuring times were standardized (Table 3). A freshly prepared set of five microsomal preparations containing different levels of cytochrome P-450 was used for this final comparison (see Materials and methods). In series three the CO-difference spectra were recorded. The protein content was determined according to Lowry (1953), also with an identical standardized protocol for each laboratory (see Materials and methods). The total cytochrome P-450 contents measured by the four laboratories are shown in Fig. 6. Although there were still small differences between the results of the laboratories, in this experiment the CV between the laboratories for the cytochrome P-450 assay (4.80%) was comparable with the CVs within the laboratories (2.51–5.55%, Table 4). The differences in the protein assays within the laboratories were small, while those observed between the laboratories were somewhat larger (Table 4).

In the fourth experiment the results of the CO- and DT-difference methods were compared. In the DT-difference method a t_{DT} of 2 min was used to allow a period of 5 min for the reaction between CO and reduced cytochrome P-450. This time span of 5 min is comparable with the 3–5 min time span found necessary in the CO-difference method (see Fig. 4). In Fig. 7 the results obtained with the CO- and DT-differences spectra in experiment four, involving three of the four laboratories, are compared. The correlation between the values obtained with both methods, using triplicate cytochrome P-450 values from the five microsome pools, was described by $y = -0.0004 + 1.088x$. The product-moment correlation coefficient according to Pearson (1981) was $\rho = 0.9861$. This correlation coefficient, found to be highly significant ($p < 0.001$) by using Students' *t*-test, indicated that the values obtained with the DT-difference method are consistently higher. This is most probably due to a higher millimolar extinction coefficient for the dithionite difference spectrum. Matsubara et al. (1976) proposed that for the calculation of the total cytochrome P-450 concentration from the DT-difference spectra a millimolar extinction coefficient of 104 $mM^{-1}.cm^{-1}$ should be used. This value is higher than the millimolar extinction coefficient of 91 $mM^{-1}.cm^{-1}$ used for calculation of the total cytochrome P-450 concentration from the CO-difference spectrum (Omura and Sato 1964b). Based on a millimolar extinction coefficient of 91 $mM^{-1}.cm^{-1}$ for the CO-difference method, from our results a millimolar extinction coefficient of 99 $mM^{-1}.cm^{-1}$ for the DT-difference spectrum can be calculated.

Finally, we found that in both methods the differential extinction remained constant with measuring times longer than 7 min (up to 20 min, results not shown). Therefore, a measuring time of 7 min is regarded as a minimum.

Summarizing, it is concluded from this study that the introduction of a strictly standardized and optimized protocol for the cytochrome P-450 and protein assays resulted in the four laboratories producing values with interlaboratory variations within the limits of intralaboratory variations. Furthermore, due to the use of this standard protocol, in future a direct comparison can be made between cytochrome P-450 and protein contents determined in toxicological experiments carried out by the four participating laboratories.

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